IN VITRO INHIBITION OF RAT LIVER TRYPTOPHAN OXYGENASE BY 4-HYDROXYPYRAZOLE

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1. Introduction

We have shown [1] that pyrazole administration to rats inhibits the hepatic activity of tryptophan oxygenase (EC 1.13.11.11). The lack of inhibitory effect on tryptophan oxygenase shown by pyrazole when added to liver homogenates together with the delayed nature of its effect in vivo suggests that the inactivator of tryptophan oxygenase is an active pyrazole derivative, as suggested [2] during studies concerned with pyrazole action on catalase (EC 1.11.1.6), another hemoprotein enzyme.

It has been shown [3,4] that the major metabolite of pyrazole in rats is 4-hydroxypyrazole and that 4-hydroxypyrazole is a potent inhibitor of catalase in vitro.

It seemed therefore of interest to test whether 4-hydroxypyrazole affects tryptophan oxygenase activity.

The present results show that 4-hydroxypyrazole inhibits tryptophan oxygenase activity in vitro, the inhibition being competitive with respect to tryptophan.

2. Materials and methods

Female Wistar rats (150 ± 5 g) maintained on a standard laboratory diet and fasted during the overnight period immediately preceding the experiments, were used.

4-Hydroxypyrazole was from Eli Lilly Co. (Indianapolis). Hemin (crystalline, of bovine origin), cortisol-21-acetate and L-tryptophan were from Sigma (St Louis).

The action of 4-hydroxypyrazole on tryptophan oxygenase activities was studied in liver homogenates prepared from cortisol-treated rats. Such rats were chosen in order to obtain liver homogenates having an elevated tryptophan oxygenase level. Cortisol-21-acetate (10 mg/rat) was injected intraperitoneally and the animals sacrificed by decapitation 4 h after treatment. The whole liver was immediately exposed and frozen exactly 1 min after decapitation. Tryptophan oxygenase activity was determined by the method in [5] with the following minor modifications. The livers were homogenized in 9 vol. 0.15 M KCl containing L-tryptophan (3×10^{-4} M) and NaOH (2.5×10^{-3} M). The homogenates were centrifuged (9000 × *g*) at 4°C for 20 min. 4-Hydroxypyrazole was dissolved in the sodium phosphate buffer and added at the start of the incubation. Preliminary experiments having shown that ascorbate interferes during kynurenine determination in the presence of 4-hydroxypyrazole, ascorbate was omitted during this incubation. The enzyme activity was expressed as micromoles of kynurenine formed per hour and per gram wet weight.

3. Results

Figure 1 shows the effects of varying the 4-hydroxypyrazole concentration on tryptophan oxygenase activities. It appears that, at <0.1 mM, 4-hydroxypyrazole activates the enzyme, while a

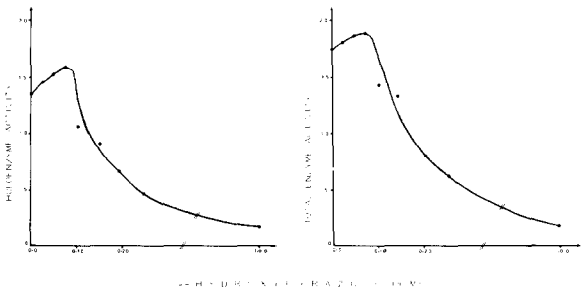

Fig.1. Effects of varying the concentration of 4-hydroxypyrazole added in vitro to tryptophan oxygenase activities in rat liver homogenates. Tryptophan oxygenase activity was determined, as in section 2, either in the absence (holoenzyme activity) or in the presence (total enzyme activity) of added hematin (5 μM). The enzyme activity is expressed as μmol kynurenine formed h/g liver wet wt. Each point represents the mean value for 8 animals.

dose-dependent sigmoidal decrease in the activities of the enzyme is observed at higher 4-hydroxypyrazole concentrations. Such effects are observed on holoenzyme as well as total enzyme activities. The inhibition of total enzyme cannot be reversed by increasing the added hematin concentration (results not shown).

The tryptophan oxygenase assay, which measures the appearance of kynurenine (and not formylkynurenine), depends on the presence of an excess of kynurenine formamidase. With formylkynurenine concentration being measured at A_{321} it was found that the A_{365}/A_{321} remained constant at all 4-hydroxypyrazole concentrations tested. It appears thus that 4-hydroxypyrazole does not lead to a relative accumulation of formylkynurenine.

A Lineweaver-Burk plot shows that 4-hydroxypyrazole acts as a competitive inhibitor with respect to tryptophan (fig.2), the K_i being 3×10^{-5} M and the K_m (tryptophan) 5×10^{-4} M.

Additional studies were designed to investigate the possible interaction of two or more sites on tryptophan oxygenase when varying tryptophan and 4-hydroxypyrazole concentrations. The arithmetical plot of velocity versus tryptophan concentration is shown fig.3. The log of the fractional activation of tryptophan oxygenase ($\log v/V-v$), when plotted against the log of tryptophan concentration, gives a maximum estimate for 'n' in the Hill equation of 1.47 (fig.3); this result is in good agreement with that in

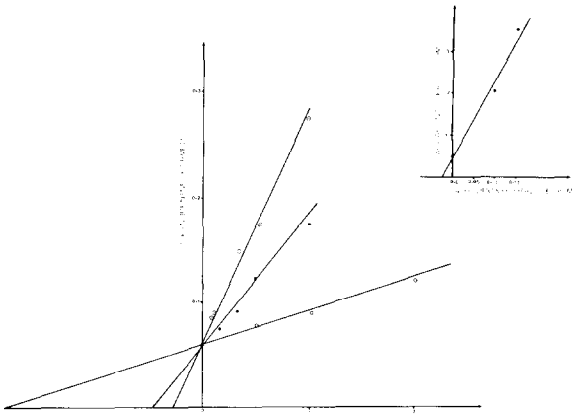

Fig.2. Lineweaver-Burk plot of inhibition of tryptophan oxygenase by 4-hydroxypyrazole, with tryptophan as the variable substrate. Enzyme activity was determined in absence of added hematin (holoenzyme activity). Each point represents the mean value for 4 animals. The curves are as follows: (–○—○–) without 4-hydroxypyrazole; (–●—●–) with 0.10 mM 4-hydroxypyrazole; (–⊙—⊙–) with 0.15 mM 4-hydroxypyrazole. The inset plots the app. K_m against the concentration of 4-hydroxypyrazole. The intercept on the X-axis gives an estimate of K_i.

[6]. Similar studies were done with 4-hydroxypyrazole at 0.025–0.25 mM, two concentrations of tryptophan (1 mM and 3 mM) being used. Log–log plots of fractional velocity versus 4-hydroxypyrazole concentration are presented in fig.4. They show that a

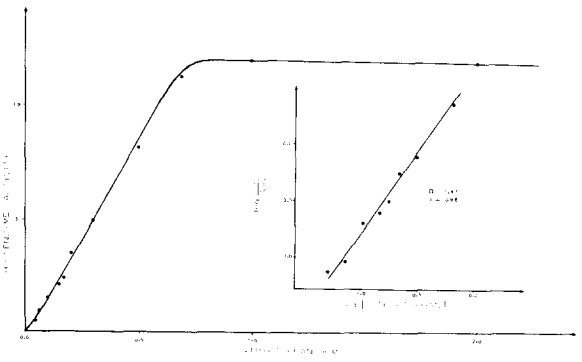

Fig.3. Tryptophan oxygenase activity as a function of the concentration of L-tryptophan. The enzyme activity was determined in absence of added hematin. Log–log plot of fractional velocity versus L-tryptophan concentration are presented in the inset. Each point represents the mean value for 4 animals. Linear regression was used to fit the line through the data points.

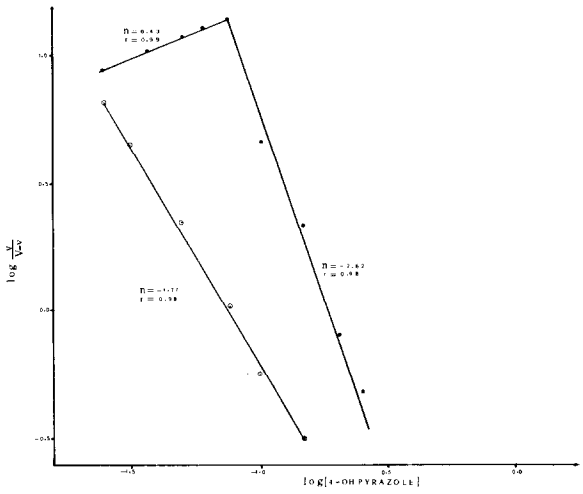

Fig.4. Hill plot of activatory and inhibitory effects of 4-hydroxypyrazole on liver tryptophan oxygenase. Only holoenzyme activity is determined. (—⊙—⊙—) Curve is with 1 mM tryptophan and varying concentration of 4-hydroxypyrazole; (—●—●—) curve is with 3 mM tryptophan. Linear regression was used to fit the line through the data points.

negative slope with $n = 1.77$ is found at 1 mM L-tryptophan, whereas, at 3 mM L-tryptophan, an initial positive slope ($n = 0.43$) is followed by a negative slope (with $n = 2.62$) when the concentration of 4-hydroxypyrazole is increased.

4. Discussion

The results obtained show that 4-hydroxypyrazole is in vitro a potent inhibitor of tryptophan oxygenase, 1 mM determining a 90% inhibition of the enzyme activities. The fact that the K_i value is several times lower than the K_m value for tryptophan is indicative of its potency. This inhibitory activity seems, however, independent of the hematin concentration.

The data for 4-hydroxypyrazole suggests that the drug interferes competitively with the binding of tryptophan. Since the Hill coefficient of 4-hydroxypyrazole increases with tryptophan concentrations (fig.4), tryptophan exhibits heterotropic interactions on the binding of 4-hydroxypyrazole. The mechanism of the activation of the enzyme which has been observed only when using a saturating tryptophan concentration and 4-hydroxypyrazole at <0.1 mM is unclear.

Our data suggest furthermore that the inhibition of tryptophan oxygenase found after pyrazole administration to rats [1] may be mediated through the formation of 4-hydroxypyrazole, which appears to have a much stronger inhibitory effect on tryptophan oxygenase activities than pyrazole itself. As a matter of fact, we found [1] that doses of pyrazole at <1 mM were without effect on tryptophan oxygenase in vitro, an inhibition of only 30% being observed when pyrazole is raised to 10 mM. Apparently the hydroxysubstitution on the ring determines this potent inhibitory behaviour. Epinephrine and other phenols are also potent inhibitors of tryptophan oxygenase in vitro and the contribution of the hydroxyl group has been pointed out [7].

The effects of pyrazole and 4-hydroxypyrazole on tryptophan oxygenase are closely similar to those described for catalase [2,4], another hemoprotein and for dopamine β-hydroxylase (EC 1.14.2.1) [8–10], a copper protein. It appeared that the presence of a hydroxyl group on the pyrazole ring determines an inhibition of these metal-containing enzymes.

Acknowledgements

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